

A probable mechanism of inactivation by urea of goat spleen cathepsin B

Unfolding and refolding studies

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Equilibrium and kinetic studies of the unfolding–refolding of goat spleen cathepsin B induced by urea are reported. Tryptophan fluorescence and enzyme activity were monitored. The activity of cathepsin B is lost reversibly at 1.2 M-urea. The enzyme unfolds in two main stages, having a stable intermediate (Y) between its native (N) and fully denatured (D) states. Enzyme activity and kinetic studies of these transitions indicate the existence of at least two intermediate forms (X_1 and X_2) between the N and Y states. The overall denaturation and renaturation scheme is thus suggested to be $N \rightleftharpoons X_1 \rightarrow X_2 \rightleftharpoons Y \rightleftharpoons D$. The multiplicity of the intermediate and fractional regaining of the activity up to a urea concentration of 2 M indicates the presence of multidomain structure in cathepsin B.

INTRODUCTION

Cathepsin B (EC 3.4.22.1) is the best known and characterized of the lysosomal cysteine proteinases (Barrett, 1977; Barrett & Kirschke, 1981). It is probably important in normal and abnormal intracellular protein turnover (Barrett, 1977). The physiological role of this enzyme is thought to be that of degradation of tissue proteins within lysosomes (Quinn & Judah, 1978; Docherty *et al.*, 1982). This cysteine proteinase is believed to be involved in several pathological conditions (Poole *et al.*, 1978; Honn *et al.*, 1982; Ostensen *et al.*, 1983), and its ability to catalyse unique modifications of several proteins has been demonstrated (Aronson & Barrett, 1978; Bond & Barrett, 1980). The enzyme acts either as a peptidyl dipeptidase or as an endopeptidase (McDonald & Ellis, 1975; Aronson & Barrett, 1978; Nakai *et al.*, 1978; Barrett & Kirschke, 1981). The exact role of cathepsin B *in vivo* is, however, still speculative.

The complete amino acid sequence of cathepsin B from rat liver has been determined by Takio *et al.* (1983). Out of seven tryptophan residues four are located in the C-terminal region and the remaining three are in the N-terminal region. Fourteen cysteine residues are distributed throughout the protein molecule, but there is no evidence that this proteinase contains any disulphide bond (Takio *et al.*, 1983).

Our present understanding of the unfolding–refolding behaviour of cathepsins is very limited, although Lah *et al.* (1984) have reported that bovine spleen cathepsin D unfolds in two main stages involving at least two intermediate forms between native and unfolded states. However, we have purified cathepsin B from a new source (goat spleen) and reported some of its various properties in previous papers (Agarwal & Khan, 1987a,b; Agarwal, 1988). In the present work we extend our studies to examine the possible intermediate conformational states during unfolding and refolding of cathepsin B in the presence of urea. Further, studies have

also been made of the dependence of enzyme activity on the concentration of urea. The results clearly show that the loss of catheptic activity is not only reversible at 1.2 M-urea but is also partially reversible up to a urea concentration of 2 M. To the best of our knowledge this is the first report of its kind and assumes especial significance as it suggests the presence of domain structure in cathepsin B.

MATERIALS AND METHODS

Materials

Spleens of freshly slaughtered goats were collected from the local slaughterhouse in ice and were processed immediately. Dimethyl sulphoxide, α -N-benzoyl-DL-arginine 2-naphthylamide (Bz-Arg-NH-Nap), 2-mercaptoethanol and EDTA were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Chromatographic media and Blue Dextran 2000 were from Pharmacia, Uppsala, Sweden. Urea (BDH Chemicals, Bombay, India) was used after recrystallization according to a published procedure (Waheed *et al.*, 1977). All other chemicals were of analytical grade.

Isolation and assay of cathepsin B

Cathepsin B was isolated and purified from goat spleen by a modification of the method of Fazili & Qasim (1986). This procedure is similar to one that we have described previously (Agarwal & Khan, 1987a). The assay of Bz-Arg-NH-Nap hydrolase activity of the enzyme at 37 °C in 20 mM-sodium phosphate buffer, pH 6.5, containing 2 mM each of EDTA and 2-mercaptoethanol was done fluorimetrically as described previously (Khan *et al.*, 1986). The effect of urea on the enzyme activity was studied by incubating a fixed amount of enzyme with different concentrations of urea in the above-mentioned buffer at 37 °C. After a 30 min incubation, the reaction was initiated by adding substrate

Abbreviation used: Bz-Arg-NH-Nap, α -N-benzoyl-DL-arginine 2-naphthylamide.

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Bz-Arg-NH-Nap (1 mg/ml) in the same buffer, and the released 2-naphthylamine was monitored continuously for 30 min with a spectrofluorophotometer. Enzyme concentration was determined by the method of Bradford (1976), with bovine serum albumin as standard.

Fluorescence

Fluorescence was measured at 37 °C with a Shimadzu RF-540 spectrofluorophotometer. The excitation and emission wavelengths were 335 and 410 nm respectively (Khan *et al.*, 1986). An excitation wavelength of 280 nm and excitation bandwidth of 5 nm were used for spectra and unfolding-refolding experiments.

Difference fluorescence spectra

The difference fluorescence spectra of cathepsin B were recorded in 20 mM-sodium phosphate buffer, pH 6.5, by using microprocessor files of the fluorimeter. Emission spectra of the enzyme solution in the presence and in the absence of urea, of solvent buffer and of urea solutions were recorded in the wavelength region 300–450 nm and stored in the files of the microprocessor. Finally, each difference spectrum was obtained by subtracting the emission spectra of native enzyme (stored previously after subtraction of the spectrum of buffer solvent) and of the urea solution from that of the spectrum of the denatured enzyme.

Equilibrium and kinetic studies

Denaturation experiments were performed by diluting 0.1 ml of stock enzyme solution with different volumes of buffer and standard urea solutions in such a way that the final volume of the mixture became 1 ml. The contents were thoroughly mixed and left for 10–12 h for equilibrium. The solutions for renaturation experiments were prepared similarly, except that the enzyme solution was first denatured with the required concentration of urea for a period sufficient for the attainment of equilibrium. In kinetic experiments the changes in emission intensity were monitored within 15 s of the addition of protein to the denaturant. First-order rate constants from biphasic processes were calculated as described by Frost & Pearson (1961).

RESULTS AND DISCUSSION

Goat spleen cathepsin B used in this study was found to be homogeneous with respect to both size and charge (Agarwal & Khan, 1987a).

Fluorescence difference spectra

Cathepsin B exhibited a single emission band at 334 nm (spectrum not shown) with no evidence of a significant contribution from tyrosine (Teale, 1960). This implies that there is extensive transfer of electronic excitation energy from tyrosine to tryptophan in the native enzyme (Konav, 1967). An increase in fluorescence intensity and a red-shift of about 14 nm in the emission maximum were observed when the enzyme was denatured in 6.4 M-urea. The difference fluorescence spectra (denatured minus native) of cathepsin B thus obtained at different urea concentrations (0.4–6.4 M) are shown in Fig. 1. As can be seen, the changes in the emission intensity were found to be maximum at 322 and 372 nm; the latter was used for monitoring the conformational changes in the enzyme since the changes in the spectral

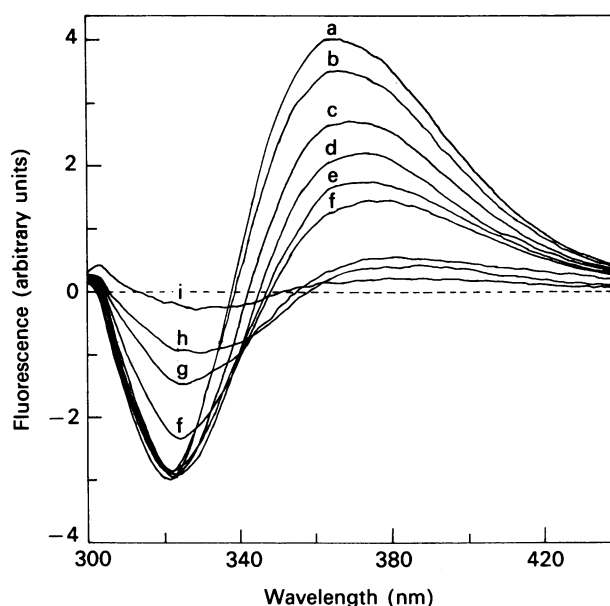


Fig. 1. Difference fluorescence-emission spectra of goat spleen cathepsin B at 37 °C

The difference spectra were obtained after subtracting the emission spectra of native enzyme and urea solutions in 20 mM-sodium phosphate buffer, pH 6.5, from the spectra of the enzyme in buffer containing urea at 6.4 M (spectrum a), 5.6 M (spectrum b), 4.4 M (spectrum c), 3.6 M (spectrum d), 2.8 M (spectrum e), 2.0 M (spectrum f), 1.2 M (spectrum g), 0.8 M (spectrum h) and 0.4 M (spectrum i). The protein concentration was 0.14 mg/ml.

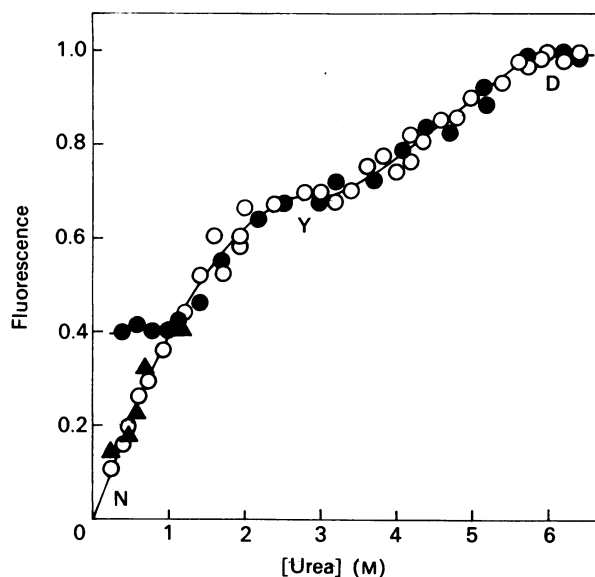


Fig. 2. Urea-induced unfolding and refolding transitions of goat spleen cathepsin B measured by fluorescence at 372 nm

○ symbols represent denaturation experiments and ● and ▲ symbols represent renaturation experiments performed after exposure of the enzyme respectively to 6.4 M- and 1.2 M-urea overnight. For the definition of N, Y and D see the text. All solutions contained 0.07 mg of protein/ml in 20 mM-sodium phosphate buffer, pH 6.5, and measurements were made at 37 °C. The excitation wavelength was 280 nm; bandwidths for excitation and emission were 5 nm.

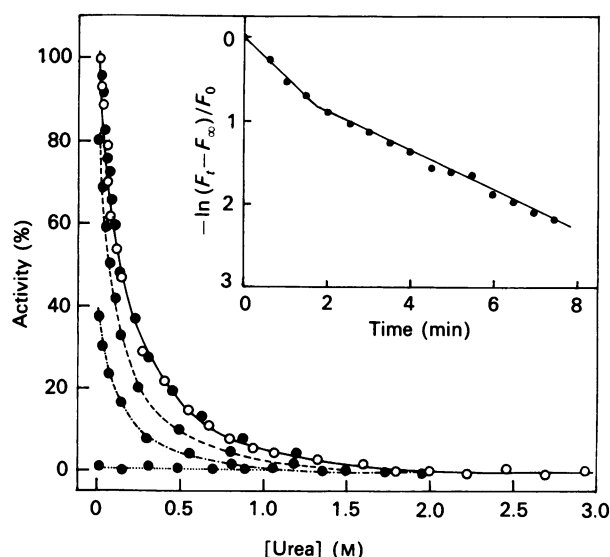


Fig. 3. Effect of urea (○) on the activity of goat spleen cathepsin B at 37 °C in 20 mM-sodium phosphate buffer, pH 6.5, containing 2 mM each of EDTA and 2-mercaptoethanol

● symbols represent the regaining of activity after exposure of the enzyme to 1.2 M-urea (—), 1.5 M-urea (---), 2.0 M-urea (· · · · ·) and at or above 2.5 M-urea (· · · · ·) overnight. Enzyme activity in the absence of urea was taken as 100%. The concentration of enzyme in each case was 7 μg/ml. The inset shows a first-order kinetic plot of the decay of cathepsin B activity in 6.4 M-urea.

properties at 322 nm were insignificant at urea concentrations above 2.8 M.

Urea-induced unfolding

Urea-induced conformational transition of goat spleen cathepsin B was measured by fluorescence measurements at 37 °C and at a wavelength of 372 nm. The results are depicted in Fig. 2. It is clear from the transition curve that the unfolding of the enzyme started at 0.2 M-urea and proceeded in two steps involving three distinguishable stable states, namely the native (N), intermediate (Y) and the fully denatured (D) states. Forms Y and D obtain at urea concentrations of 2.8 M and 5.6 M respectively. The reversibility of the transition was checked at different concentrations of urea after dilution of the enzyme solution that was exposed to 6.4 M-urea. The denaturation curve was found to be reversible until the urea concentration was lowered to 1.3 M, whether the initial urea concentration was 6.4 M or 2.5 M. When the urea concentration was lowered to below 1.3 M, the transition was irreversible (Fig. 2, ● symbols). Interestingly, this phase of the transition was reversible when the initial concentration of urea was 1.2 M (Fig. 2, ▲ symbols). These results suggested the possibility of the presence of one or more intermediates during the unfolding transitions of cathepsin B (Tanford, 1968).

Inactivation by urea

The activity of cathepsin B at increasing concentration of urea was studied at pH 6.5 at 37 °C, and a plot of percentage activity versus urea concentration is shown in Fig. 3. It is evident from the curve that more than 40% of the activity of the enzyme was lost at urea concentrations as low as 0.1 M; the activity is completely

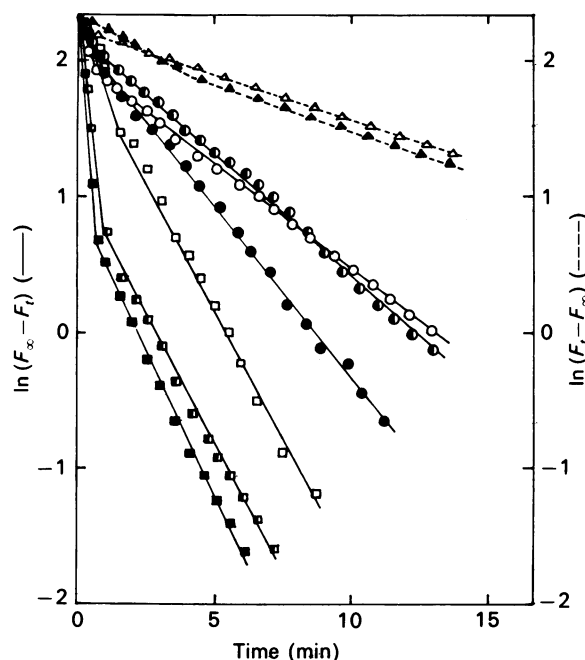


Fig. 4. Kinetics of unfolding of goat spleen cathepsin B by urea monitored by fluorescence emission at 372 nm (—) and 322 nm (---) in 20 mM-sodium phosphate buffer, pH 6.5, at 37 °C

Fluorescence emission (F) was used to monitor the unfolding of the enzyme in 1 M-urea or below (Δ), 1.2 M-urea (▲), 2 M-urea (○), 2.8 M-urea (●), 3.6 M-urea (◐), 4.4 M-urea (◑), 5.6 M-urea (◒) and 6.4 M-urea (◓). The protein concentration was 0.14 mg/ml. Results are plotted according to first-order kinetics.

inhibited at urea concentrations of 1.2 M and above. The reversibility of the enzyme inactivation was also measured after exposing cathepsin B at various concentrations of urea and adjusting back to low concentrations of urea by dilution. The loss of enzyme activity was found to be completely reversible when the enzyme was exposed to urea concentrations up to 1.2 M. However, the percentage recovery of the activity decreased when the enzyme was exposed to increasing concentrations of urea (Fig. 3); the inactivation became completely irreversible at urea concentrations of 2.5 M or above. It should be pointed out that the denaturation curve (after exposure to 6.4 M-urea) also shows a tendency to irreversibility at nearly the same urea concentration (approx. 1.3 M) at which the enzyme lost its activity completely. Furthermore, regaining of partial activity up to 2 M-urea indicates the possibility of the existence of some additional intermediate(s) at urea concentrations between 1.2 M and 2 M.

Kinetics of inactivation

The inactivation of goat spleen cathepsin B at 37 °C in 20 mM-sodium phosphate buffer, pH 6.5, containing 6.4 M-urea was studied with respect to time. A steady decrease in the enzyme activity was observed (results not shown); and more than 90% activity was abolished within 8 min. A first-order plot of the kinetic results of enzyme inactivation is shown in the inset to Fig. 3. The appearance of a biphasic curve having rate constants of 0.402 min^{-1} and 0.235 min^{-1} points to the involvement of a kinetic intermediate in the pathway of the transition.

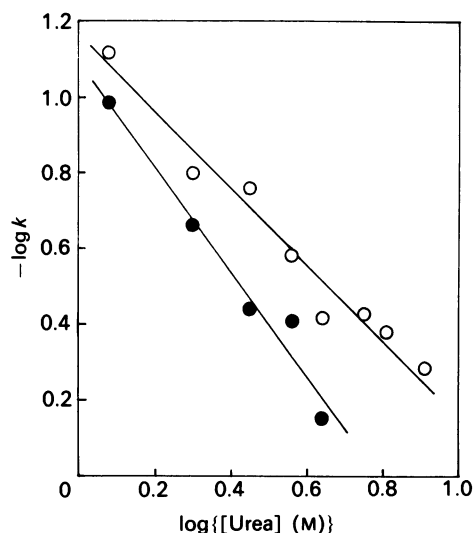


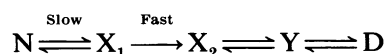
Fig. 5. Dependence of the rate constants of the unfolding of cathepsin B on urea concentration

Biphasic rate plots were decomposed to give first-order rate constants for the fast (●) and the slow (○) phases. The lines for the rate constants are least-squares-regression slopes for the values obtained from fluorescence experiments.

Kinetics of denaturation

The progress of denaturation of the enzyme was followed by monitoring the fluorescence emission at 322 and 372 nm. The first-order rate plots were found to be monophasic up to an urea concentration of 1 M; the plots were biphasic at urea concentrations of 1.2 M and above (Fig. 4). The biphasic kinetics with the fast phase taking over at higher urea concentrations and a strong dependence of the rate constants of the two phases (fast and slow) on urea concentration (Fig. 5) not only rule out the possibility of *cis-trans* isomerization due to proline residues (Brandts *et al.*, 1975) but also reflect the presence of an intermediate species in the unfolding pathway at 1.2 M-urea. The fact that both phases are positively dependent on urea concentration indicates that the intermediate species has a conformation intermediate between those of the native (N) and partially unfolded (Y) states (Ikai & Tanford, 1973). The values of $d(\log k)/d(\log [\text{urea}])$ obtained by least-squares analysis of the fluorescence results are 1.0 and 1.4 respectively for the slow and the fast phases.

In view of the above experimental observations, the mechanism of goat spleen cathepsin B unfolding and its inactivation with urea can be expressed by the following simple model:



where form X_1 exists at 1.2 M-urea, form X_2 is transient, form Y exists at 2.8 M-urea and D is the fully denatured protein. Since the protein was excited at 280 nm and the fluorescence changes significantly (approx. 40%) between 0 and 1.2 M-urea (Fig. 2), the reversible transition $\text{N} \rightleftharpoons \text{X}_1$ probably consists in a conformational alteration involving perturbation of the environment of tryptophan and/or tyrosine residues. Further, an active cysteine residue at position 29 is located near a tryptophan residue

(Takio *et al.*, 1983), and the perturbation of the latter might be a cause for the enzyme inactivation. Transitions $\text{X}_2 \rightleftharpoons \text{Y}$ and $\text{Y} \rightleftharpoons \text{D}$ represent the major conformational change in the protein (Fig. 2). As has been mentioned, the X_2 form is transient, and a detailed study of the transitions $\text{X}_1 \rightarrow \text{X}_2 \rightleftharpoons \text{Y}$ would require time-resolved techniques. The fact that the transition $\text{X}_1 \rightarrow \text{X}_2$ is irreversible may be due to the inhibition of refolding by post-translational modifications (Erickson *et al.*, 1981; Hasilik *et al.*, 1982). Also, we cannot exclude the possibility that lysosomal proteinases originate from precursors by proteolytic cleavages (Hasilik & Neufeld, 1980; Erickson *et al.*, 1981; Hasilik & von Figura, 1984). In these general properties goat spleen cathepsin B is quite different from bovine spleen cathepsin D, in which activity is lost irreversibly on unfolding with guanidinium chloride (Lah *et al.*, 1984). However, it seems to be similar to pepsin (Ahmad & McPhie, 1978; Privalov *et al.*, 1981), which possesses a marked domain structure (Andreeva & Gustchina, 1979).

Thus the urea-induced unfolding of cathepsin B seems to be a very complex process. There may be some kinetic and equilibrium intermediate on the unfolding-refolding pathway of the enzyme additional to what we have discussed above. Since the possibility of a non-specific intermediate involving some kind of thiol-disulphide exchange was ruled out by testing the aggregation properties of the enzyme, the multiplicity of the intermediates therefore might be taken as an indication for the presence of a multidomain structure in cathepsin B. However, more data are required to test this possibility unequivocally.

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